# SOME ASPECTS OF PROTEIN DEGRADATION IN LEATHER PROCESSING

## INTRODUCTION

Animal hides and skins have been used by man before recorded time. These prehistoric men must have found that freshly flayed skins will decompose rapidly if not protected. Means of accomplishing this were found, chiefly by drying and treatment with salt, and these methods, with refinements, are still in use today.

The skin of an animal is a highly complex structure. Figure 1 is a diagrammatic representation of a cross section of a steerhide and the complexity is clearly seen. During the processing into leather many of these components are removed and those remaining are more or less altered. When a hide or skin is in a condition ready for tanning, it consists primarily of the fibrous protein collagen. However, residues of such other components as elastin, reticulin, muscle fibers, mucopolysaccharides, fat, and some cellular material still remain.

Some of the degradative changes which take place during curing and depilation will be discussed.

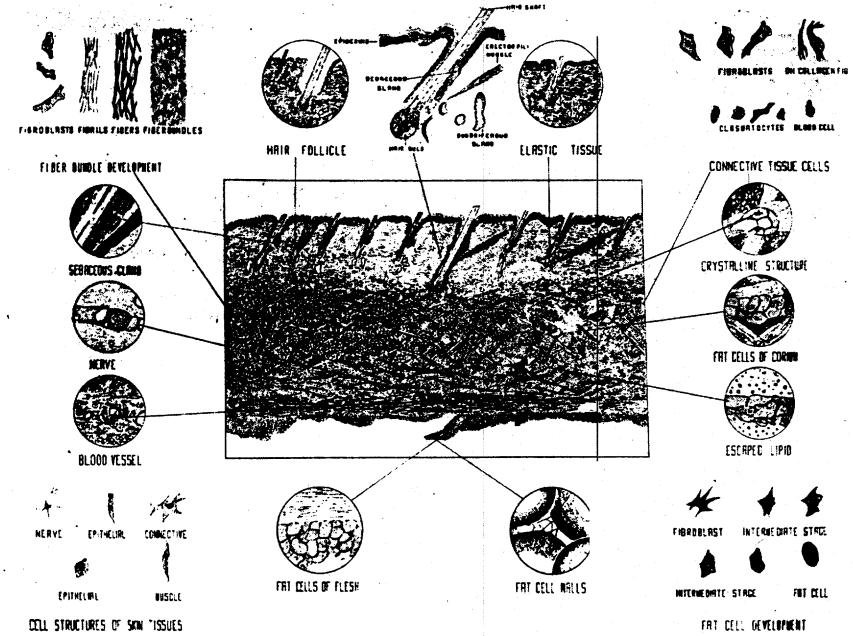


Fig. 1. Diagram of a cross section of steerhide showing its complex y. (Courtesy of William Roddy, University of Cincinnati.)

Table 1. Salt Tolerance and Collagen Solubilizing Activity of Representative Single Cultures (from Everett and Cordon, 1956)

| Cultures | Growth without Salt | Growth with 4M Salt(23%) | Activity* in 15% Salt Broth + Substrate | | |
|---|---|---|---|---|---|
| | | | 12% Gelatin | Limed+ Collagen | Unlimed‡ Collagen |
| Uninoculated controls | - | - | 0 | 10-20% | 3-9% |
| Gram-pos. rods: | | | | | |
| No. 12 | Yes | No | +++ | 62 | 8 |
| No. 11 | Yes | Yes | +++ | 32 | 5 |
| No. 87 | No | Yes | ++ | 34 | 6 |
| Gram-neg. rods: | | | | | |
| No. 21 | Yes | Yes | +++ | 41 | 5 |
| No. 22 | Yes | Yes | +++ | 36 | 3 |
| No. 20 | No | Yes | ++ | 20 | 4 |
| Gram-pos. cocci: | | | | | |
| No. 28 | No | Yes | ++ | 98 | 5 |
| No. 78 | No | Yes | ++ | 31 | 7 |
| No. 59 | Yes | Yes | + | 25 | 7 |
| Gram-var. red cocci: | | | | | |
| No. 112 | No | Yes | + | 20 | 6 |
| Gram-var. irreg. rods: | | | | | |
| No. 104 | No | Yes | ++ | 20 | 10 |

*Gelatin, complete liquefaction: +++ in 4 weeks; ++ 4 to 8 weeks; + over 8 weeks. Collagen, solubilization: hydroxyproline in solution as per cent of total found.

+Center layer of cowhide soaked in saturated lime liquor; chemically sterilized.

‡Center layer of cowhide carefully purified without liming; chemically sterilized.

## CURING

Treatment of hides with common salt is the usual procedure for curing. Salt reacts with the moisture in the hide and produces a brine which dissolves some of the globular proteins along with some of the constituents of blood. This process also reduces the moisture level of the hide from 60-65% to about 45-50%. This lowered moisture, together with the high salt content, prevents the growth of most bacteria and other microorganisms. Some halophilic bacteria are able to develop, however, particularly during extended periods of storage and may cause staining, hair slip, or decomposition of the collagen.

We were interested in determining what types of bacteria were present on salt-cured hide and what effect pure cultures would have on collagen (Everett, A. L. and Cordon, T. C., 1956). During the course of the study, several hundred cultures of bacteria were isolated from salt-cured hides, brines, etc., and grown on high-salt media. They were tested for their ability to liquefy gelatin and the positive ones were used for studies on decomposition of collagen.

Collagen was prepared in essentially unaltered condition by splitting off the grain and flesh layers and using only the center portion. This is known as the corium split. Small pieces of this material were sterilized by the method of Maxwell (1945). Five to ten gram lots of this material were treated in succession in closed containers, with 100 ml of each of the following solutions: (1) 0.025 M so-

dium metabisulfite for 17 hr; (2) 0.3 M hydrogen peroxide for 7 hr; (3) sterile 0.1 M sodium bicarbonate for 17 hr; (4) two changes of sterile 10% salt. The bisulfite solution was at pH 2.0 and the first three solutions contained 10% salt to prevent swelling.

Small pieces of the sterilized collagen were placed in tubes containing a complete broth medium and incubated at 30 C for several weeks to check sterility and then inoculated with test cultures. After two to three months of incubation the solid material was filtered off, both fractions were hydrolyzed by autoclaving in 6N HCl in sealed tubes, and hydroxyproline determined by the method of Neuman and Logan (1950). Since this amino acid is unique to collagen this is an effective measure of collagen breakdown.

Table 1 shows the results with some representative cultures. Several of them required salt for growth, all but one could grow in a 23% salt solution, all attacked gelatin more or less strongly, and most made some attack on limed collagen; several attacked it very extensively. None of these pure cultures was able to attack unlimed collagen.

Results with mixtures of these pure cultures are given in Table 2. Here again, limed collagen was attacked by most of the combinations but only a few were able

Table 2. Enhanced Activity Shown by Various Mixed Cultures (from Everett and Cordon, 1956)

| Composition of Culture Mixtures | Total No. of strains | Activity* in 15% Salt Broth + Substrate | |
|---|---|---|---|
| | | Limed† Collagen | Unlimed‡ Collagen |
| Uninoculated controls | 0 | 10-20% | 3-9% |
| A Gram-pos. rods | 19 | 47 | 10 |
| B Gram-neg. rods | 5 | 32 | 5 |
| C Gram-pos. cocci | 30 | 40 | 5 |
| D Gram-var. red cocci | 10 | 15 | 8 |
| E Gram-var. irreg. rods | 3 | 42 | 8 |
| Group Combinations | | | |
| A + B | 24 | 65 | 8 |
| A + C | 49 | 29 | - |
| A + D | 29 | 22 | - |
| A + E | 22 | 41 | 11 |
| B + C | 35 | 37 | 5 |
| B + D | 15 | 21 | 6 |
| B + E | 8 | 91 | 11 |
| C + D | 40 | 24 | - |
| C + E | 33 | 62 | 14 |
| D + E | 13 | 58 | 8 |
| A + B + C + D + E | 67 | 93 | 15 |
| Selected Mixtures | | | |
| No. 19 (B) + E | 4 | 88 | 36 |
| No. 104 (E) + B | 6 | 93 | 40 |

*Collagen solubilization: hydroxyproline in solution as per cent of total found.
†Center layer of cowhide soaked in saturated lime liquor; chemically sterilized.
‡Center layer of cowhide carefully purified without liming; chemically sterilized.

to degrade the unlimed collagen. These results were surprising in view of the degradation which sometimes takes place in salt-cured hides. It appears that suitable combinations of organisms under favorable growth conditions may be required to effect this type of action. The effect of mild hydrolysis of collagen by liming on its susceptibility to microbial attack is clearly shown.

## UNHAIRING

The almost universal method of removing the hair from animal hides consists of a treatment with saturated lime containing a "sharpening" agent, such as sodium sulfide, arsenic sulfide, sodium sulfhydrate, or amines. We have just seen how treatment of collagen with lime renders it susceptible to microbial attack. Hormann and Schubert (1958) have shown that treatment of collagen with lime causes some solubilization of a component which is rich in hydroxyproline. Fifteen percent of the nonammoniacal nitrogen in the solubilized portion is hydroxyproline, compared with only 6.9% in whole collagen. It appears that a hydroxyproline-rich portion of the molecule must have been attacked. This lime-treated collagen was subjected to the action of trypsin and compared with unlimed material. Trypsin was able to attack the limed but not the unlimed material.

Grassmann and Hannig (1958) have studied the effect of the denaturation of collagen by mild heat on its susceptibility to attack by trypsin and chymotrypsin. Both of these enzymes were able to attack the heated collagen but not the unheated. Here again a mild denaturation has resulted in making collagen susceptible to the action of biological agents.

In order to alleviate waste disposal, shorten the processing time, and produce a better leather, we have been investigating the depilatory action of enzymes on hides and skins. We found very soon that freshly flayed hides were not nearly so readily unhaired by enzymes as were salt-cured hides or hides which had been soaked in dilute salt solution. Beuchler and Lollar (1949) have shown that salt alone has a depilatory action under certain conditions. If fresh hide is allowed to stand for several days in a 2-10% salt solution containing a disinfectant to prevent microbial growth, the entire epidermis becomes loosened and can be removed intact. Unfortunately, it is not loose enough for commercial unhairing. Figure 2 shows a piece of intact epidermis being removed and Fig. 3 shows the intact epidermis as it appears under the microscope. Note that the epidermal lining continues around the hair roots and that the sebaceous or oil glands are part of this system. Parts of the sweat glands can often be seen in such preparations.

We were very much interested in learning how salt made hides more susceptible to enzyme action and employed histological techniques to help elucidate this phenomenon. Burton, Reed, and Flint (1953) have postulated that the binding material which holds the epidermis to the dermis, sometimes called the basement membrane, is a mucopolysaccharide. We cut thin sections from untreated hide pieces or those soaked in water or salt solutions, and stained them with the periodic acid-Schiff stain which visualizes carbohydrate material. Figure 4 consists of photomicrographs of some of these sections. The section of untreated hide shows an intensely stained band at the juncture of the dermis and epidermis. After soaking in water some of the background staining material is removed, but the intense band

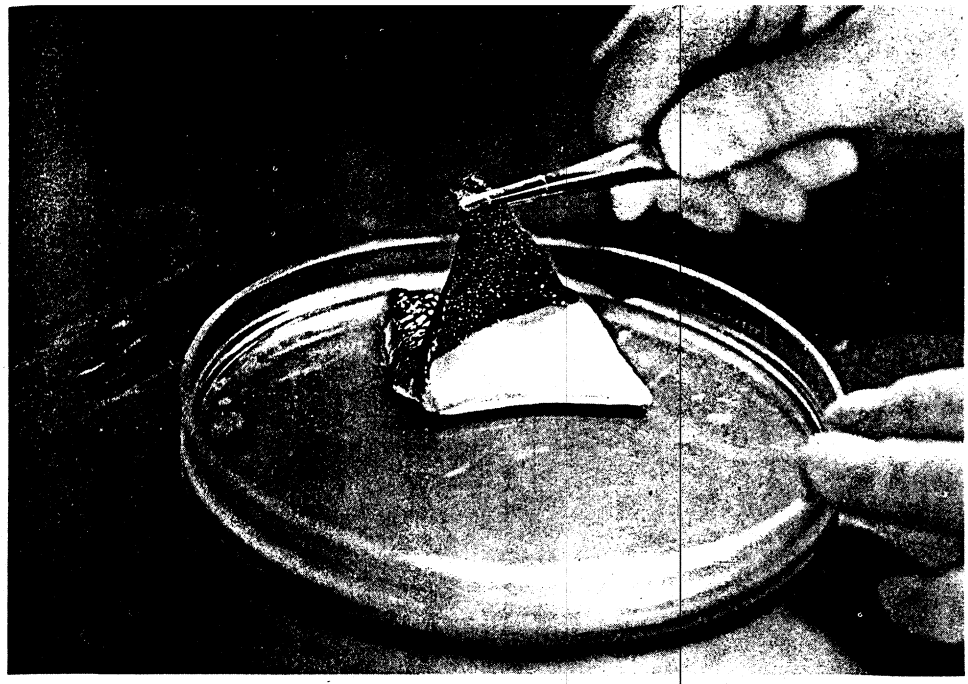

Fig. 2. Removal of intact epidermis from steerhide after soaking in dilute salt water.

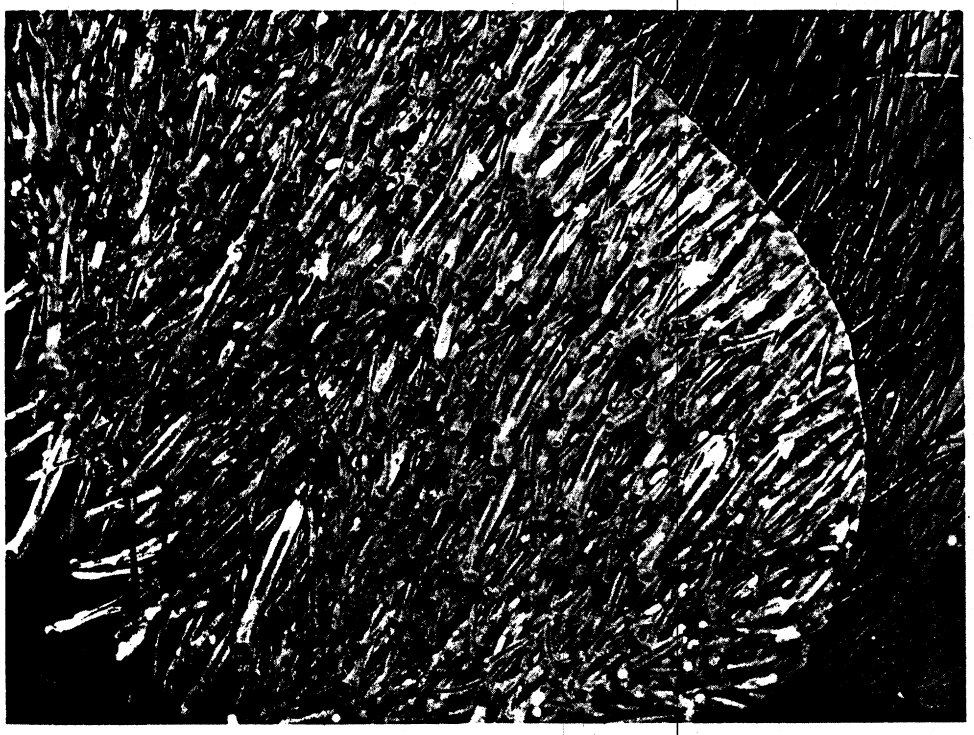

Fig. 3. Intact epidermis from steerhide showing details of its structure. (From Everett and Cordon, 1959.)

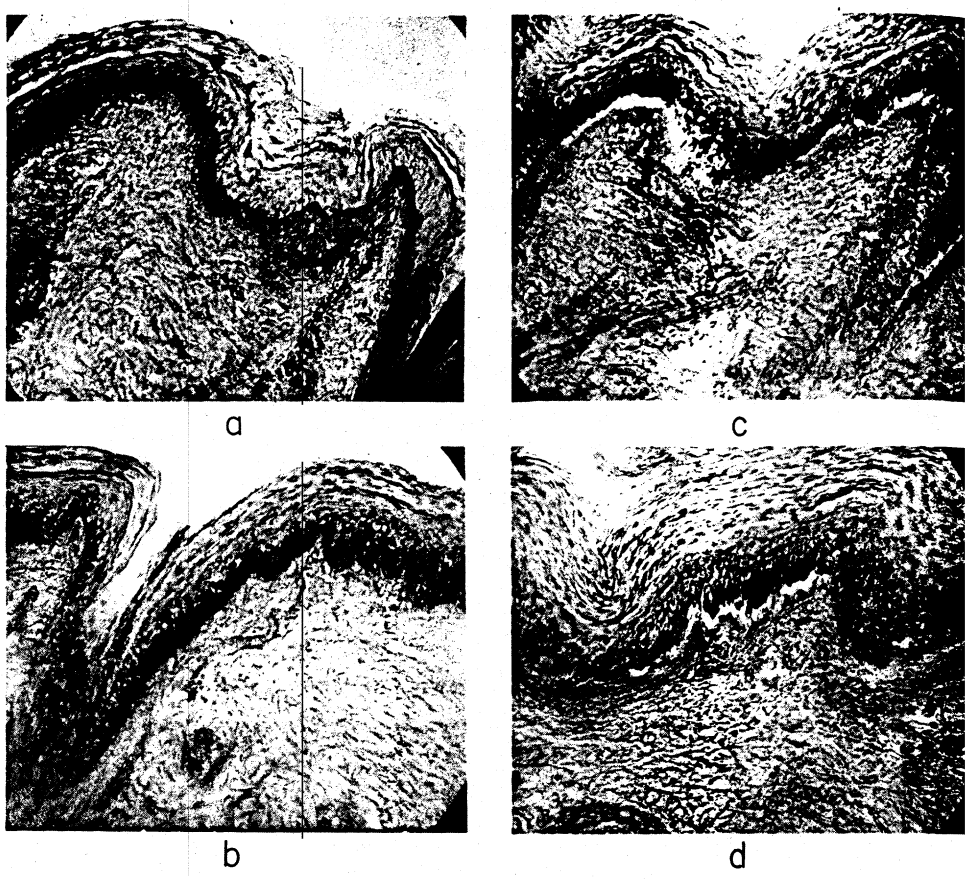


Fig. 4. Cross sections of fresh hide, PAS stain. X200. a) untreated; b) soaked in water; c) soaked in 4.8% salt water; d) soaked in 9.1% salt water. (From Everett and Cordon, 1958.)

still remains. After soaking in dilute salt the intensely stained layer is much more diffuse and the epidermis has separated from the dermis in some places. After treatment with depilatory enzymes this carbohydrate-staining material is completely removed. We believe that salt treatment causes a peptization or partial degradation of the bonding layer and thus conditions it for enzyme action.

As another approach to uncover the nature of the material which holds the epidermis to the dermis, we have tried to correlate the depilatory action of various enzymes with assays on known substrates (Cordon, T. C. *et al.*, 1958). The data in Table 3 illustrate the results. Assay values for action on casein (PV), gelatin (formol titration), and starch (DV), are tabulated so that any relation to hair loosening could be observed. For several enzymes we have also measured gelatinase activity as a function of lowering the viscosity and keratinase activity on scoured wool. None of these activities showed a direct correlation with hair-loosening action, but in general enzymes showing good depilatory action also have high proteolytic activity.

Table 3. Relation of Hair Loosening to Other Activities of Some Selected Enzyme Preparations (from Cordon *et al.*, 1959)

| Enzyme | PV(a) Units/g | Formol Titration(b) meq-N/g | DV(c) Units/g | Concentration Used g/100 ml | Hair Looseness P(d) R(e) |
|---|---|---|---|---|---|
| HT Concentrate(f) | 190,000 | 46.0 | 95,000 | 0.1 | 7-84 |
| HT Proteolytic(f) | 331,000 | 41.8 | 9,200 | 0.06 | 7-83 |
| L-56D(g) | 100,000 | 35.5 | 4,500 | 0.19 | 7-84 |
| Protease 15 Concentrate(h) | 162,000 | 26.7 | 2,200 | 0.12 | 8-77 |
| 4511-3(i) | 46,300 | 19.1 | 17,000 | 0.41 | 9-76 |
| Papain | 24,800 | 10.0 | <300 | 0.77 | 3-97 |
| Bromelin | 46,200 | 46.3 | <400 | 0.41 | 4-91 |
| Pancreatin 3 XUSP | 66,800 | 36.6 | 16,300 | 0.28 | 10-25 |
| Trypsin 4X USP Pancreatin | 66,500 | 38.6 | 3,200 | 0.29 | 10-0 |

(a) Action on casein.
(b) Action on gelatin.
(c) Starch-dextrinizing power.
(d) Number of pulls with scraper. See Fig. 1.
(e) Estimation of percent of hair removed.
(f) Bacterial enzymes from the Takamine Laboratories.
(g) Bacterial enzyme from Pabst Laboratories.
(h) Bacterial enzyme from Röhm & Haas Company.
(i) Bacterial enzyme from Wallerstein Company.

The fibrous protein elastin has been suggested as being implicated in bonding the epidermis to the dermis and with this in mind we have assayed a number of depilatory enzymes for elastase activity. Figure 5 shows photographs of cross sections of hide after enzyme treatment and staining with the Fullmer-Lillie elastin stain. The elastin fibers are stained black and run roughly parallel to the surface of the hide. In some cases enzyme treatment completely removes these fibers.

Some of the enzymes which showed strong depilatory action also show strong elastase activity but here again the relationship to hair loosening is only casual and no direct correlation could be made. It appears that a mucopolysaccharide material may be involved in cementing the epidermis to the dermis; at least such a material is present before but not after the action of depilatory enzymes. Such enzymes also degrade elastin but no direct relation to hair loosening has been proved.

## SUMMARY

It is concluded that during the processing of hides and skins in preparation for tanning many degradative processes take place. Some of these are necessary for the removal of unwanted constituents and some are required to condition the hide for tanning.

Some of these changes are undoubtedly brought about by halophilic bacteria existing in the salt-cured hides during storage. This represents a complex problem of microbial ecology. For it has been shown that undenatured collagen is very

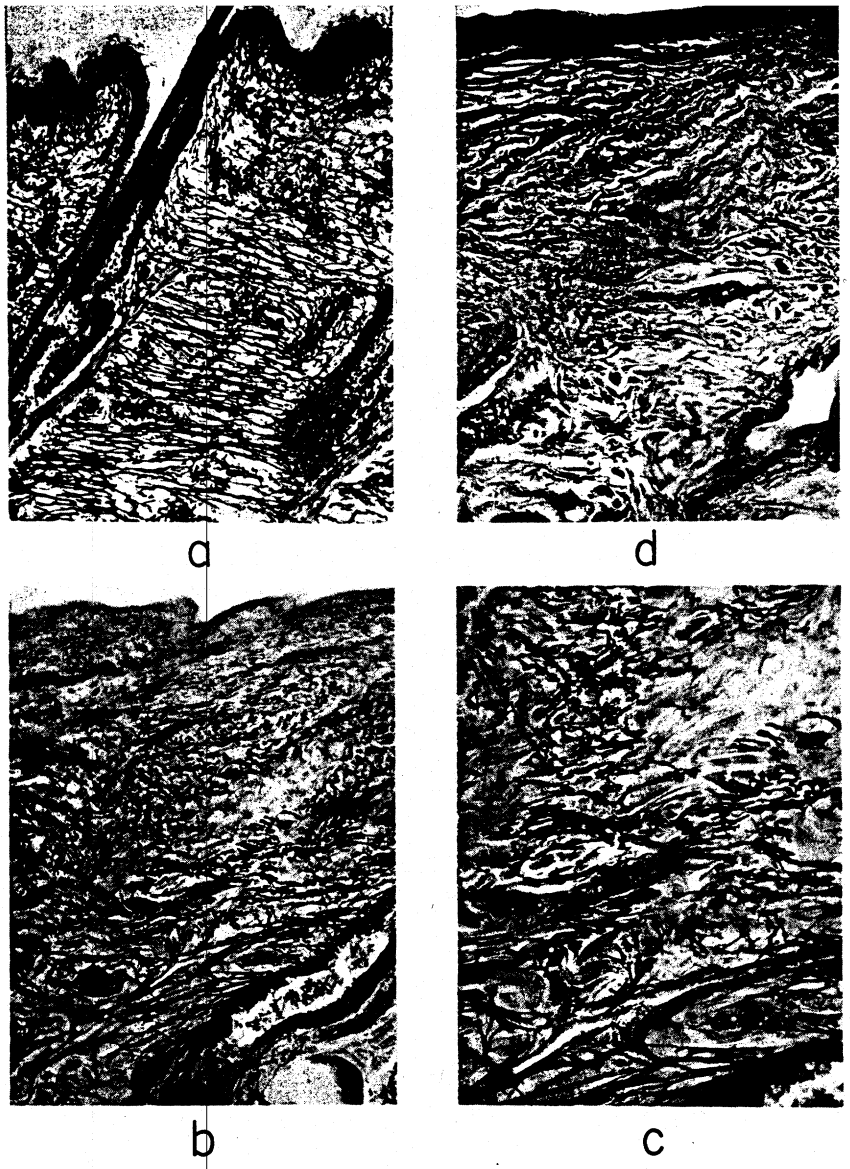


Fig. 5. Cross section of steerhide, stained for elastin by Fullmer-Lillie method; magnification 100× for a, b, and d, 200× for c. a) untreated control; b and c) treated with papain solution for about 7 hr. Illustrates incomplete removal of elastin. d) treated with papain solution overnight. Illustrates almost complete removal of elastin. (From Everett et al., 1959.)

resistant to attack by single pure cultures, while degradation could be obtained with certain mixtures. However, collagen that has been denatured by treatment with lime, is susceptible to attack even by single cultures. Similarly, trypsin and chymotrypsin can degrade heat-denatured or lime-treated collagen but not native collagen.

## REFERENCES


Beuchler, P. R., and Lollar, R. M. 1949 Some sulfur distribution studies on cattle hair and epidermis. J. Am. Leather Chem. Assoc., 44, 359-370.

Burton, D., Reed, R., and Flint, F. O. 1953 The unhairing of hides and skins without lime and sulfide. The use of mucolytic enzymes. J. Am. Leather Trades' Chemists, 37, 82-87.

Cordon, T. C., Jones, H. W., Clarke, I. D., and Naghski, J. 1958 Microbial and other enzymes as depilatory agents. Appl. Microbiol., 6, 293-297.

Cordon, T. C., Windus, W., Clark, I. D., and Naghski, J. 1959 A progress report on the enzyme depilation of cattle hides. J. Am. Leather Chem. Assoc., 54, 122-139.

Everett, A. L., and Cordon, T. C. 1956 Resistance of purified collagen to degradation by salt-tolerant bacteria in pure culture. J. Am. Leather Chem. Assoc., 51, 59-55.

Everett, A. L., and Cordon, T. C. 1958 The use of salt to assist enzymatic unhairing of fresh hides, with histochemical observations. J. Am. Leather Chem. Assoc., 53, 548-567.

Everett, A. L., Cordon, T. C., Kravitz, E., and Naghski, J. 1959 *In situ* histological evaluation of elastase activity. Stain Technology, 34 (6), 325-330.

Grassmann, W., and Hannig, K. Cited in Hormann and Schubert, Das Leder 9.

Hormann, H., and Schubert, B. 1958 Chemical changes of hide substance under liming and bating conditions. Das Leder 9, 265-274.

Maxwell, M. E. 1945 Fellmongering investigations. IV Bacteria responsible for the loosening of wool on sheepskins. C.S.I.R. Australia, Bull. No. 184, 89-116.

Neuman, R. E., and Logan, M. A. 1950 The determination of hydroxyproline. J. Biol. Chem. 184, 299-306.